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Isolation of Proteins Interacting with the Cyclin D1-CDK6 Complex from Normal and Tumorigenic Human Breast Cells Using a Novel Yeast Three-Hybrid System

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A hallmark of many tumor cells is their ability to continuously cycle under conditions where normal cells would be proliferating at a reduced rate. Transitions through different cell cycle stages are primarily regulated by a family of closely related protein kinases, the cyclin-dependent kinases (CDKs), presumably by phosphorylating critical cellular proteins

Cyclin D1 is a proto-oncogene found to be amplified in 15-20% of human breast cancer cases. Cyclin D1, through its association with the catalytic subunit CDK6 and CDK4, controls G1 cell cycle progression, presumably by phosphorylating a substrate protein(s) whose function is involved in controlling cell growth and whose activity is critically regulated by the cyclin D1-dependent kinases in G1.

Here I describe the development of a yeast three-hybrid system that expresses two separate genes from the same plasmid and potentially supports the formation of a ternary complex in the yeast cell. Using this system, I have screened a human keratinocyte cDNA library to assay the ability of the three-hybrid system to identify proteins known to interact with CDK6/cyclin D1. In addition to the proteins known to interact with this complex (p130 and p21), several novel interactions were detected, some of which were phosphorylated by the complex in vitro.

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I. Introduction

A hallmark of many tumor cells is their ability to continuously cycle under conditions where normal cells would either be quiescent or proliferating at a reduced rate. Therefore, the molecular pathways controlling the cell cycle must inevitably interact with pathways which regulate cell growth and suppress tumorigenesis. Eukaryotic cell cycle progression is composed of multiple transitions between different cell cycle states. One of the most important transitions is during late G1, defined as START in yeast or the restriction (R) point in mammalian cells. Until this point, cells are sensitive to a wide variety of growth regulation, including extracellular growth factors, DNA damage, and inducers of cellular differentiation. Once past the START or R point, cells become largely refractory to these regulations and are committed to complete a new round of DNA replication. Deregulation of the START or R checkpoint may therefore allow cells to bypass normal restrictions on entry into S phase and promote an uncontrolled growth phenotype characteristic of tumor cells.

Biochemically, transitions through different cell cycle stages are primarily regulated by a family of closely related protein kinases, the cyclin-dependent kinases (CDKs), presumably by phosphorylating critical cellular proteins. The activity of CDKs are regulated at three different levels: binding with an activating subunit, a cyclin; subunit phosphorylation, and inhibition by a CDK inhibitor (Morgan, 1995; Peter and Herskowitz, 1994; Sherr and Roberts, 1995). Both CDK and cyclins constitute a multigene family, different members presumably functioning to regulate different stages of the cell cycle through their combinatorial interactions (Figure 1). The cyclins that are most closely linked to the regulation of mammalian START are the D-type cyclin-dependent CDK4 and CDK6 (Figure 1). Mammalian cyclin D1 was initially isolated by virtue of its ability to rescue yeast cells deficient for G1 cyclin function [CLN1, CLN2 and CLN3, (Xiong et al., 1991)], its induction in response to colony-stimulating factor 1 (Matsushime et al., 1991), and in a search for the putative oncogene *PRAD1* rearranged in parathyroid tumors (Motokura et al., 1991). Cyclin D1 is amplified in 15-20% of human breast cancers as part of the 11q13 amplicon and is also genetically linked to the *bcl-1* oncogene, a locus activated by translocation to an immunoglobulin gene enhancer in some B cell lymphomas and leukemias. Examination of human mammary tumors for the expression of the 11q13 locus indicated that the cyclin D1 gene, but not other proto-oncogenes such as *INT2* and *HST1*, is located at the position where a relevant oncogene is expected, based on detailed Southern analysis, and was found to be consistently amplified in breast cancer cells (Motokura and Arnold, 1993; Lammie and Peters, 1991; Withers et al., 1991). Transgenic expression of cyclin D1 in mammary tissue lead to the development of mammary hyperplasia and carcinoma (Wang et al., 1994). Mice lacking cyclin D1 fail to undergo the massive proliferation of the mammary epithelial compartment associated with pregnancy (Sicinski et al., 1995). The molecular mechanism by which altered cyclin D1 expression contributes to breast cancer development is not clear. One hypothesis is that the amplification of cyclin D1 leads to the elevated kinase activity of cyclin D1-CDK4/CDK6, resulting in inappropriate phosphorylation of cyclin D1 targeted substrate protein(s) whose function is involved in controlling mammary cell growth and whose activity is critically regulated by the cyclin D1-dependent kinases in G1. Thus far, the only substrate of cyclin D-CDK4/6 kinases that has been identified is the retinoblastoma susceptibility gene product, pRb whose growth suppressing activity is negatively regulated by the CDKs (DeCaprio et al., 1989; Chen et al., 1989). Using a novel three-hybrid system that I have recently developed, I propose to address this critical issue by identifying cellular protein(s) that specifically interact with the cyclin D1-CDK6 complex in normal mammary epithelial cells (HMECs) and breast cancer cells.

The major negative regulation of CDKs in mammalian cells is accomplished by CDK inhibitors (Sherr and Roberts, 1995). Two distinct families of CDK inhibitors, represented by two prototype CDK inhibitors, p21 and p16, have been identified in mammalian cells. p21 was first discovered in normal human fibroblast cells as a component of cyclin D-CDK quaternary complexes that also contain proliferating cell nuclear antigen [PCNA, (Xiong et al., 1992)]. *p21*

(also known as *WAF1, CIP1, SDI1, PIC1*, CAP20) encodes a potent inhibitor of multiple cyclin-CDK enzymes. The expression of p21 is transcriptionally activated by the tumor suppresser p53 (El-Deiry et al., 1993). Mice lacking p21 are defective in G1 checkpoint control when exposed to gamma-irradiation (Deng et al., 1995; Brugarolas et al., 1995; Waldman et al., 1995). The second member of this family, p27, has recently been shown to be a direct target of the viral oncoprotein E1A (Mal et al., 1996), suggesting a potential mechanism by which a viral oncoprotein deregulates normal cell cycle control. Mice lacking p27 display multi-organ hyperplasia, suggesting that this protein is necessary for normal growth control (Okuda et al., 1995; Nakayama et al., 1996; Kiyokawa et al., 1996). p16/INK4a (also known as MTS1, CDK4I) was first observed as a CDK4-associated protein in human cells (Xiong et al., 1993) and was subsequently cloned and characterized as a specific inhibitor of the CDK4/6-cyclin D kinases (Serrano et al., 1993). Three additional members of the INK4 family have since been isolated: p15*INK4b* (Kamb et al., 1994; Hannon and Beach, 1994; Guan et al., 1994), p18*INK4c* (Guan et al., 1994; Hirai et al., 1995) and p19*INK4d* (Chan et al., 1995; Hirai et al., 1995; Guan et al., 1996). p16 and its neighboring p15, have been found to be mutated or deleted in many human tumor derived cell lines as well as human primary tumors (Sherr and Roberts, 1995). p16 is now believed to represent the first melanoma susceptibility gene, located on chromosome 9p21. p16 null mice develop multiple tumors at an early age, further suggesting a role for p16 as a tumor suppresser (Serrano et al., 1996), but this interpretation has been complicated due to the recent discovery of a second gene, p19 *ARF* (Alternative Reading Frame), occupying the same locus. p19 *ARF* was discovered as a gene that shares the second exon of p16, albeit in a different reading frame. Splicing of the first exon of ARF to the second exon of p16, results in a protein with no homology to any known CDK inhibitor(Kamijo et al., 1997). Interaction of members of the p21 inhibitor family with CDKs is stimulated by or dependent on the cyclin subunit, suggesting that the p21 family of inhibitors preferentially interact with cyclin-CDK complexes (Guan et al., 1996; Harper et al., 1995). As an example, human p27*Kip1* was isolated using CDK4 as bait in combination with cyclin D1 (Toyoshima and Hunter, 1994). Co-expression of cyclin D1 and CDK6 using the yeast three-hybrid vector may allow me to identify potential novel members of the p21 CDK inhibitor family. In addition, expression of some CDK inhibitor genes, in particular members of the p16 family, exhibit remarkable tissue specificity (Guan et al., 1994; Guan et al., 1996). A screen specific for CDK4 and CDK6 interacting proteins has not previously been conducted in normal HMECs or breast cancer cells.

Hypothesis and Purpose

The yeast two-hybrid system is a method for detecting protein-protein interactions (Fields and Song, 1989). The assay takes advantage of the modular nature of the GAL4 transcription factor which contains separable DNA binding (GAL4-BD)and transcriptional activation domains (GAL4-AD). In this system, two plasmids are introduced into yeast- one plasmid that encodes the GAL4-BD fused to the protein of interest, the "bait", and the other encoding the GAL4-AD fused to another protein, "the prey", often introduced as a cDNA library. If the bait and the prey proteins interact, the GAL4-BD and the GAL4-AD are brought into close proximity and activate expression of a nutritional reporter gene under the control of a GAL4-responsive promoter, allowing yeast cells to grow in the absence of the nutrient. Numerous protein-protein interactions have since been discovered using this system, including the interaction of the CDK inhibitor, p18*INK4c*, with human CDK6 in this laboratory (Guan et al., 1994).

A major limitation of the two-hybrid system is that only one gene can be expressed from the bait plasmid. As a result, only proteins that form a binary complex with the bait protein can be identified. It can not detect interactions that require the bait protein complexed with a second protein. This becomes critical in identifying substrates of CDK enzymes since formation of the cyclin-CDK complex is necessary to possess enzymatic activity toward its substrate. In addition, members of the p21 CDK inhibitor family are known to interact preferentially, if not exclusively,

with the cyclin-CDK complexes. Here the P.I. describes the development of a yeast three-hybrid system (Figure 2) that allows the co-expression of the GAL4 BD-bait (CDK6) fusion and a second gene (cyclin D1), which then form a complex capable of interacting with the GAL4 AD-prey fusion. This approach will be used to screen both a normal HMEC library as well as a human breast cancer library. Identification of cellular protein(s) interacting with the cyclin D1-CDK6 complex and any changes between normal and transformed cells may lend insight into the process of cellular transformation associated with breast cancer.

II. Body

To conduct a yeast three-hybrid screen, two vectors, pGBT6 and pGBT7 (Figure 3), were constructed (see figure legend for details).

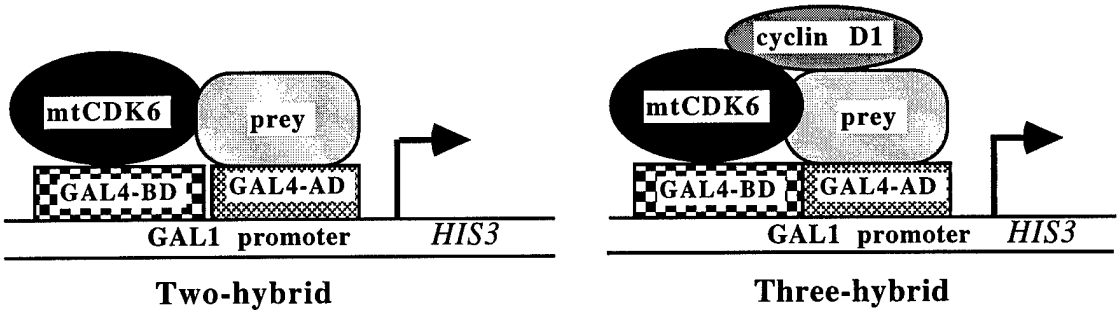

Fig. 2. Schematic comparison of yeast two-hybrid and three-hybrid systems.

Catalytically inactive CDK4(K35M), but not wild type, in association with its partner cyclin D1 forms a stable ternary complex with its substrate pRb (Kato et al., 1993). In order to stabilize the substrate interaction with cyclin D1-CDK6, I have constructed a similar catalytically inactive CDK6 mutant, mtCDK6, by mutating the lysine residue at codon 43 to methionine (K43M) in the ATP binding domain through PCR mediated site-directed mutagenesis. This mutant CDK6 cDNA was fused in-frame with the GAL4-BD in both pGBT6 and pGBT7. The human cyclin D1 was inserted with (pGBT7-mtCDK6/D1) or without (pGBT6-mtCDK6/D1) fusion with the NLS. Both constructs were introduced into the yeast strain HF7c (*his3-200, leu2-3, trp1-901*). To test the three-hybrid system, prey plasmids encoding two types of Cyclin D1-CDK6 interacting proteins were transformed into Hf7c in combination with either pGBT6-mtCDK6/D1, pGBT7-mtCDK6/D1, or pGBT7-mtCDK6.

In the first assay, the C-terminus of pRb that is commonly used as the substrate of *in vitro* kinase assays for cyclin D-CDK4 and cyclin D-CDK6 enzymes was fused in-frame with the GAL4-AD in a yeast expression plasmid, pGAD, that carries the leu marker. Yeast cells transformed with pGAD-Rb with pGBT6-mtCDK6/D1, pGBT7-mtCDK6/D1, but not with pGBT7-mtCDK6 that does not express cyclin D1, grew on media lacking leucine, tryptophan and histidine (figure 4), indicating that mtCDK6 and D1 were interacting with pRb protein and that this interaction is cyclin D1 dependent.

In the second assay, the CDK inhibitor p21 was fused in-frame with the GAL4-AD of pGAD, as described above. Yeast cells transformed with pGBT7-mCDK6/D1 and pGAD-p21 grew on media in the absence of leucine, tryptophan and histidine, while yeast transformed with pGBT7-mCDK6 and pGAD-p21 did not (figure 4). In this experiment, pGBT6-mCDK6/D1 was not assayed.

Because of the low level of leakage of the GAL1 promoter and the presence of dying yeast cells whose histidine may be secreted into the media to support the growth of false positive yeast cells, a second independent assay for the bait and prey protein interaction was carried out. Interestingly, while both pGBT7 (cyclin D1 fused with NLS) and pGBT6 (cyclin D1 synthesized

without the NLS) are capable of activating the expression of the *HIS3* gene, only pGBT7 is able to activate the expression of the *lacZ* gene (data not shown), suggesting that the nuclear localization signal enhances the interaction of cyclin D1 with CDK6(K43M) and the pRb.

Yeast Three-Hybrid Screen

The major effort of this proposal is to use the three-hybrid system to identify cellular proteins, from normal HMECs and breast cancer cells, that interact with the cyclin D1-CDK6 complex. Prior to generating the needed HMEC cDNA libraries, however, the three-hybrid system was tested for its ability to detect cyclin D1-CDK6 interacting proteins in an actual library screen. There was some concern that in actual use the three hybrid system may either primarily detect interactions with the GAL4-BD fusion protein, or that there may be an unworkably high background, due to the expression of three proteins in a single yeast cell. In order to address these concerns, an available human keratinocyte cDNA library was used to assess the ability of the three hybrid system to detect cyclin D1-CDK6 interacting proteins.

One hundred micrograms of plasmid DNA from a human keratinocyte (HaCaT) library subcloned into pACT2 was transformed separately into yeast HF7c cells pGBT7-mtCDK6/D1, pGBT7-mtCDK6/D3 or pGBT7-mtCDK6 using the lithium acetate method. After incubating on selective media at 30°C for between three and four days, positive colonies that grew on histidine, leucine and tryptophan deficient media were patched onto the same selective media to confirm their growth phenotype and then assayed for the expression of β-gal activity by a qualitative filter assay. The library plasmid insert was amplified from positive yeast colonies by PCR using a pair of oligo primers specific to the pACT2 prey plasmid. PCR products from all positive colonies were digested by a frequently cutting restriction enzyme (either Alu I or HaeIII). cDNA clones with identical digestion patterns were grouped and a representative clone from each group was sequenced. The results from these screens is summarized in Table 1.

Importantly, both p21 and the pRb family member p130 were isolated using pGBT7-mtCDK6/D1 but not pGBT7-mtCDK6. p21 is known to preferentially bind to cyclin-CDK complexes and p130 is a known substrate of the cyclin D/CDK6 complex. These results are a strong indication that the three-hybrid system is working as expected. In order to examine both the validity, specificity, and the type of interactions being detected using the three-hybrid screen, experiments were conducted to confirm the interaction in yeast, assay the interaction in a system outside of yeast, and to determine whether the isolates were indeed capable of being phosphorylated by the Cyclin D1/CDK6 complex.

Confirmation and characterization of positive clones interacting with mtCDK6/cyclin D

For most of the positive clones listed in Table 1, the library plasmid was rescued, confirmed by restriction mapping and re-transformed into Hf7c yeast. The only exceptions are those clones that were either known or suspected false positives. In addition, the positive clones were tested by two-hybrid assay for their ability to interact with cyclin D1 alone, cyclin D3 alone, mtCDK6 alone, and by three-hybrid assay for their ability to interact with the mtCDK6/cyclin D1 complex and the mtCDK6/cyclin D3 complex. As shown in figure 5, each positive clone was able to interact with cyclin D alone, but none were capable of interacting with mtCDK6 alone, indicating a cyclin D dependent interaction between isolated proteins and CDK6 and formation of a ternary CDK6-cyclin D-prey protein complex in yeast cells.

Of the proteins isolated from the two three-hybrid screens (cyclin D1/mtCDK6 and cyclin D3/mtCDK6), fibronectin receptor shows a preference for cyclin D3 over cyclin D1, while tuberin, thymidine kinase and peroxisomal enoyl- CoA-hydratase (ECH) show a preference for cyclin D1. These observations are based on figure 5 as well as the number of isolates from each screen as shown in Table 1. In addition, both tuberin and ECH interact with mtCDK6/cyclin D1 in

the presence of the *HIS3* inhibitor, 30 mM 3-amino-triazole (3-AT), indicative of a strong interaction. In contrast, tuberin and ECH interact weakly with the mtCDK6/cyclin D3 complex, as judged by poor growth on media lacking histidine and a lack of interaction detected during the library screening. These results suggest that despite a high degree of sequence similarity between cyclin D1 and D3, they may target the same catalytic CDK6 subunit to distinct substrate proteins.

As an initial examination as to whether the interacting proteins may serve as substrates for the cyclin D1-CDK6 complex, in vitro kinase assays were conducted. GST fusion proteins were incubated on beads with baculovirus extract expressing either CDK6 alone, cyclin D1 alone, or CDK6 and cyclin D1 together. Positive clones were found to be phosphorylated when incubated with both CDK6 and cyclin D1 both not with either component alone (Figure 6). In this assay, tuberin, thymidine kinase and clone H4, a novel protein, were found to be phosphorylated. ECH and TRAP1 do not appear to be phosphorylated, although it should be noted that these are not the full length proteins. Thus, although the cyclin D may interact with the C-terminus, the phosphorylation sites may reside elsewhere. The individual characteristics of the isolated genes are discussed below.

A brief summary of the genes isolated from HaCaT using CDK6/cyclin D1 or D3

Cytochrome oxidase III (cox III) is a mitochondrial gene that encodes one of the 13 subunits of cytochrome c oxidase. Levels of coxIII are abnormally low in colon tumors (Mao et al., 1995), but are inducible by metabolizable unbranched fatty acids, which enhances cytochrome c oxidase activity (Duro et al., 1995). There is no data regarding the phosphorylation state of this protein. This protein was found to interact weakly with mtCDK6/cyclin D1, but nonetheless representing 15% of all positive clones. Interestingly, coxIII was never found in association with mtCDK6/cyclin D3 (Table 1). CoxIII has not undergone any additional testing.

Thymidine kinase is an enzyme that catalyzes the phosphorylation of thymidine to thymidine monophosphate. Thymidine kinase is induced to maximal levels just prior to DNA synthesis in S phase (Quelle et al., 1997). In addition, thymidine kinase is found to exist in phosphorylated and non-phosphorylated forms with phosphorylation occurring on serine 224 of the carboxyl terminus (Leach et al., 1993). It is not clear, however, whether this phosphorylation serves to regulate the activity of thymidine kinase. Most studies have focused on the transcriptional and post-transcriptional regulation of the gene. Thymidine kinase is a 25 kD protein and the clone isolated from the three-hybrid screen is full length. Although thymidine kinase appears to associate with cyclin D1 and the CDK6/cyclin D1 complex and also appears to be phosphorylated by CDK6/cyclin D1 *in vitro*, the interaction is relatively weak in yeast when compared to other isolates. Growth on media lacking histidine is weak and β-gal assays are negative.

Peroxisomal enoyl-CoA-hydratase (ECH) is a 36 kDa protein involved in the β-oxidation of very long chain fatty acids (VLCFA). The gene encoding ECH, ECH1, is strongly induced in response to peroxisome proliferators (Chen et al., 1996). A rare X-linked recessive condition associated with abnormal β-oxidation of VLCFA is associated with by severe multi-system disease, often resulting in early death (Burnatowska-Hledin et al., 1995). There is no data in the literature to indicate that phophorylation of ECH has been examined. Based on yeast data, ECH interacts strongly with CDK6/cyclin D1 and cyclin D1. In complex with CDK6/cyclin D1 or with cyclin D1 alone, ECH grows well on media lacking histidine and in the presence of 30mM 3-AT. In qualitative β-galactosidase filter assays, ECH appears to be equally as strong or stronger than the positive controls. ECH shows a strong preference for cyclin D1 over cyclin D3 as shown by transformations with each and notably has not been identified as an interacting protein in the mtCDK6/cyclin D3 screen although it was isolated as 11% of the isolates from the mtCDK6/cyclin D1 screen.

Tuberin is the product of the TSC2 gene, which was isolated by positional cloning as a gene segregating with the autosomal dominant disease, tuberous sclerosis (Stankovic et al., 1997). This disease is characterized by the widespread development of growths in many tissues and organs. The gene is appears to be a tumor suppressor, as indicated by the association of a loss of heterozygosity in the growths of tuberous sclerosis patients (Walsh and Perlman, 1997; Yun and Wold, 1996) Northern blot analysis revealed a 5.5kb transcript present in all cell lines tested, including brain, kidney, skin, liver, adrenal gland, colon and white blood cells (Stankovic et al., 1997). The protein is 1748 amino acids with a calculated molecular mass of 198 kD. The protein has a 58 amino acid region with homology to ras family GTPase-activating proteins (Stankovic et al., 1997). In addition, the carboxyl-terminus has two transcriptional activation domains (Maione and Amati, 1997). The carboxyl terminal region (consisting of the amino acids 1163-1743) was found to localize to the nucleus while the full length was found to localize to the cytoplasm. In my initial experiments, the carboxyl terminus of tuberin was found to interact with cyclin D1 in the GST pulldown assay and to be phosphorylated by CDK6/cyclin D1. Tuberin interacts strongly with the cyclin D1 complex and cyclin D1 but weakly with the cyclin D3 complex and cyclin D3 alone.

Tissue necrosis factor receptor associated protein 1 (TRAP1) is a 56 kDa protein cloned by doing a two-hybrid screen using the tissue necrosis factor receptor type 2 as bait (Tybulewicz et al., 1991). TRAP1 mRNA is barely detectable in normal tissues but is upregulated in transformed cell lines (Tybulewicz et al., 1991). The N-terminus of TRAP1 contains both RING finger and zinc finger motifs often found in DNA binding proteins (Tybulewicz et al., 1991). There is no data in the literature indicating that the phosphorylation potential of TRAP1 has been examined. TRAP1 was isolated from HaCaT using both mtCDK6/cyclin D1 and mtCDK6/cyclin D3 as bait. The function of TRAP1 is unknown.

The fibronectin receptor is one mediator of cell adhesion via attachment to a fibronectin substrate. The fibronectin receptor β-subunit was isolated from a HaCaT library using mtCDK6/cyclin D3, but not mtCDK6/cyclin D1 as bait (Table 1). Indeed, in subsequent tests, the positive clone was negative for growth with the mtCDK6/cyclin D1 complex (fig. 5). There is an enormous amount of literature regarding this gene, although there is no information regarding its phosphorylation with regards to a serine/threonine kinase. The full-length β-subunit is 88 kDa although the positive clone isolated is truncated, perhaps explaining its weak interaction with both mtCDK6/cyclin D3 and cyclin D3 alone.

Dopachrome tautomerase (DCT) was cloned from a human melanoma cDNA library and encodes a protein of 59 kDa (Phelps et al., 1998). There is correlative evidence showing that in malignant melanocytes, DCT activity is elevated. There is no literature addressing the phosphorylation of DCT. DCT was represents 5% of the positive clones isolated from the mtCDK6/cyclin D3 screen, but was never detected in association with mtCDK6/cyclin D1 (Table 1). DCT has not undergone any additional testing, including re-transformation, but the fact that multiple isolates were observed makes it doubtful that the interaction is due to a second prey plasmid.

Succinate-ubiquinone oxidoreductase is a 30 kDa protein cloned by immunoscreening of a human liver cDNA library. This enzyme is involved in both the Krebs cycle and the aerobic respiratory chains of the mitochondria. There is limited literature regarding this gene and there is no indication as to its phosphorylation. This gene interacted weakly with mtCDK6/cyclin D3 and was never detected with mtCDK6/D1. This gene has not undergone additional testing, but does represent 5% of the positive clones analyzed from the mtCDK6/cyclin D3 screen.

III. Conclusions

This report describes the completion of the development of a yeast three-hybrid system. In addition, the system has been tested by screening a human keratinocyte cDNA library and found to be effective. This conclusion is based on two criteria: 1) Using mtCDK6/D1 as bait, but not using mtCDK6 alone, proteins known to interact with CDK6 in a cyclin D dependent fashion were isolated (e.g. p21 and p130). 2) The CDK6/cyclin D complex was shown to be capable of phosphorylating some of the isolates from the HaCat screen (e.g. Thymidine kinase and tuberin). This implies that the complex is interacting with the protein isolate, in a context outside of yeast, and also suggests some regulatory function for CDK6/cyclin D of these proteins. One caveat is that *in vitro* kinase assays are sometimes misleading, and overexpression can sometimes force a phosphorylation that is not physiologically relevant. Thus, further studies would be required before a conclusion can be drawn as to whether or not the detected interactions occur *in vivo*.

The next objective of this project is two-fold: 1) Determine whether any of the isloates from the HaCaT two-hybrid screen have any relevance to human breast cancer; 2) Generate cDNA libraries from both normal and tumorigenic HMECs to be used for a three-hybrid screen. These aspects of the project are currently underway.

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Table 1. Summary of HaCaT cDNA library screens

	Bait		
	pGBT7-mtCDK6	pGBT7-CDK6/cyc D1	pGBT7-CDK6/cyc D3
Total number screened	$1X10^6$	$1X10^6$	$2.53X10^6$
Efficiency	$1X10^4/\mu g$	$1X10^4/\mu g$	$2.5X10^4/\mu g$
Number of clones analyzed	57	135	43
Gene			
CyclinD1	65%	23%	28%
Cyclin D2	5%	3%	5%
Cyclin D3	7%	0	0
p15INK4B	12%	11%	9%
p16 INK4A	5%	1	1
p21	0	7%	16%
p130	0	2%	1
Tuberin	0	15%	5%
CoxIII	0	15%	0
Peroxisomal enoyl-CoA-hydratase	0	11%	0
Thymidine Kinase	0	5%	0
Fibronectin receptor	0	0	5%
Dopachrome tautomerase	0	0	5%
Ubiquinone	0	0	5%
Novel	4%	8%	12%
Other	2%	3%	0

III-B.4.b Illustrations/Diagrams/Chemical Syntheses

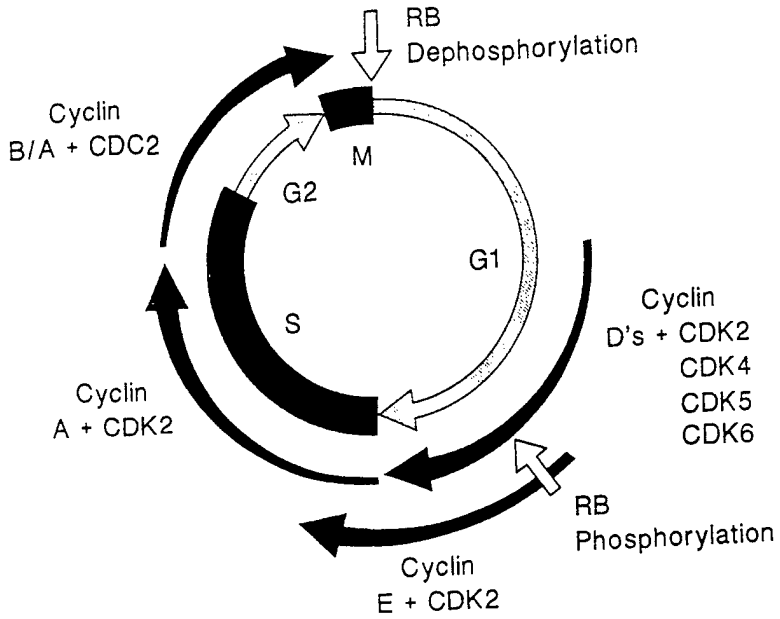

Figure 1. **General schematic of the mammalian cell cycle.**
Shown are the interactions of different cyclins and CDKs as well as the functional status of pRb. Adapted from Sherr, 1993.

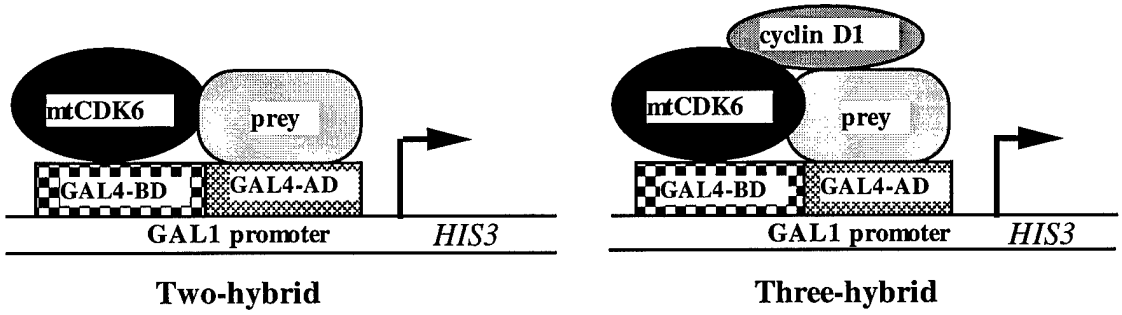

Figure 2. **Schematic comparison of the yeast two-hybrid and three-hybrid systems.**
While the two-hybrid system is suitable for detecting binary interactions, the three-hybrid is capable of detecting proteins in a ternary complex.

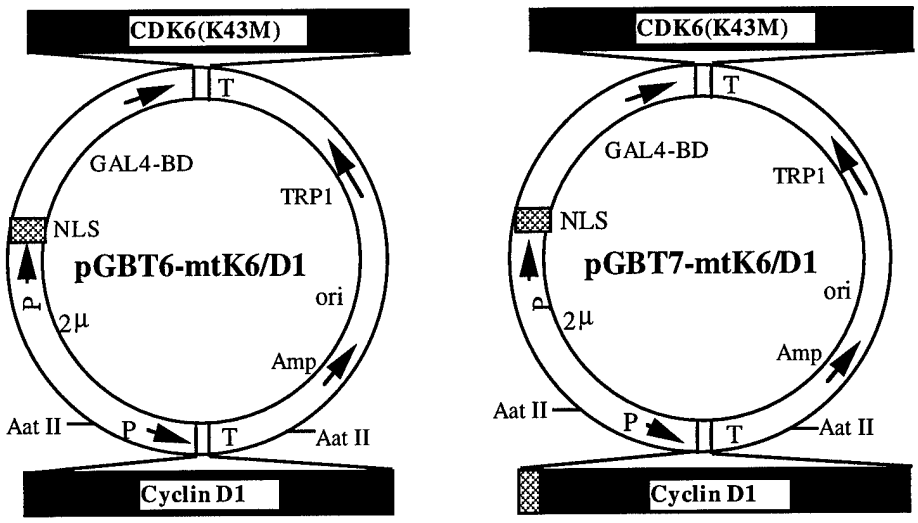

Fig. 3. **Yeast three-hybrid vectors.**
Key: P: yeast ADH promoter, T: yeast ADH terminator,
Ori: E.coli origin of replication, NLS: nuclear localization signal, GAL4-BD: GAL4 DNA binding domain, Amp: -lactamase, TRP1: tryptophan
Both plasmids were derived from pGBT8, a modified form of pGBT9 that has been widely used for yeast two-hybrid screening. In addition to the yeast 2m origin of replication, the E.coli origin of replication, and the ampicillin resistance gene, pGBT8 carries the tryptophan marker (TRP1) and a strong yeast alcohol dehydrogenase (ADH) promoter directing the expression of the yeast GAL4-BD followed by a multiple cloning sequence (MCS) for the insertion of the bait cDNA. A 915 base pair Aat II restriction fragment was generated from pGBT8 by PCR that contains an ADH promoter followed by a sequence encoding a nuclear localization signal (NLS, the first 73 amino acids of the GAL4), a unique MCS and the ADH termination sequence. This cassette was inserted into the Aat II site of pGBT8, resulting in pGBT7. A similar experimental methodology was used to generate pGBT6 that does not retain the nuclear localization signal. Both pGBT7 and pGTB6 were confirmed by restriction mapping and partial sequencing.

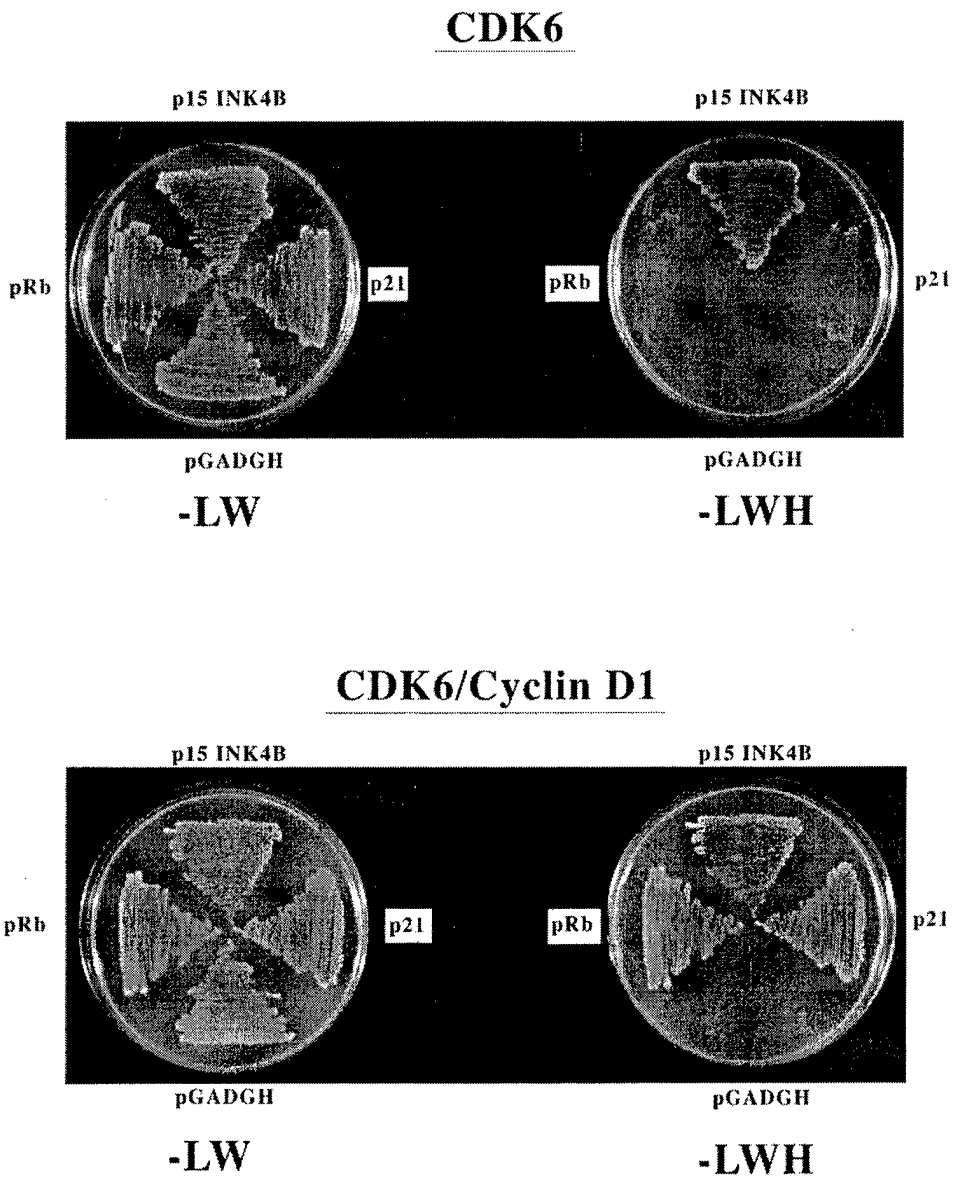

Figure 4. **pRb and p21 interact with mtCDK6 in a cyclin D dependent fashion.** Yeast cells were transformed with either pBGT7-mtCDK6 or pGBT7-mtCDK6/D1 as bait and various prey plasmids expressing the indicated gene subcloned into pGADGH. Yeast were plated onto -leucine, -tryptophan to show that both plasmids were present and that the yeast were viable. To select for yeast expressing interacting proteins, yeast were plated onto media lacking leucine, tryptophan and histidine. Plates were incubated at 30 celsius for three days and re-streaked onto fresh plates. These plates were again incubated at 30 celsius for three days. pGADGH is empty vector serving as the negative control.

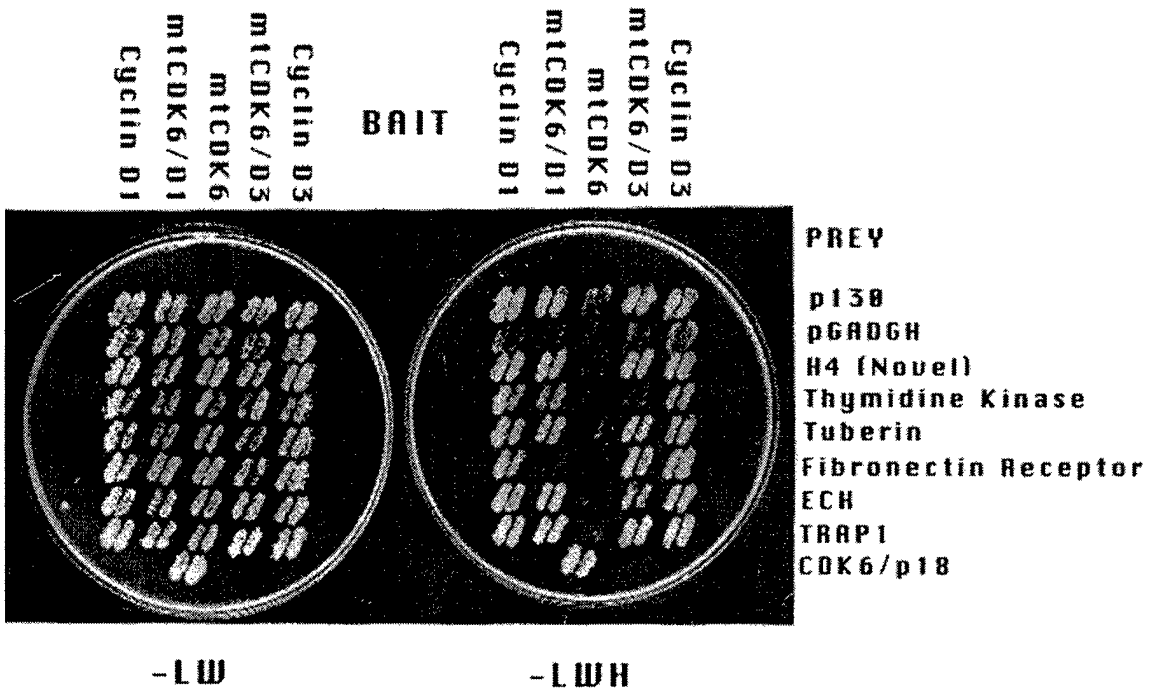

Figure 5. **Cyclin D1/ Cyclin D3 specific interaction of various isolates with mtCDK6** Yeast cells were transformed with either pBGT7-mtCDK6 or pGBT7-mtCDK6/D1 or pGBT7-mtCDK6/D3 as bait and various prey plasmids expressing the indicated gene subcloned into pGADGH or pACT2. Yeast were plated onto -leucine, -tryptophan to show that both plasmids were present and that the yeast were viable. To select for yeast expressing interacting proteins, yeast were plated onto media lacking leucine, tryptophan and histidine. Plates were incubated at 30 celsius for three days and re-streaked onto fresh plates. These plates were again incubated at 30 celsius for three days. pGADGH is empty vector, serving as the negative control.

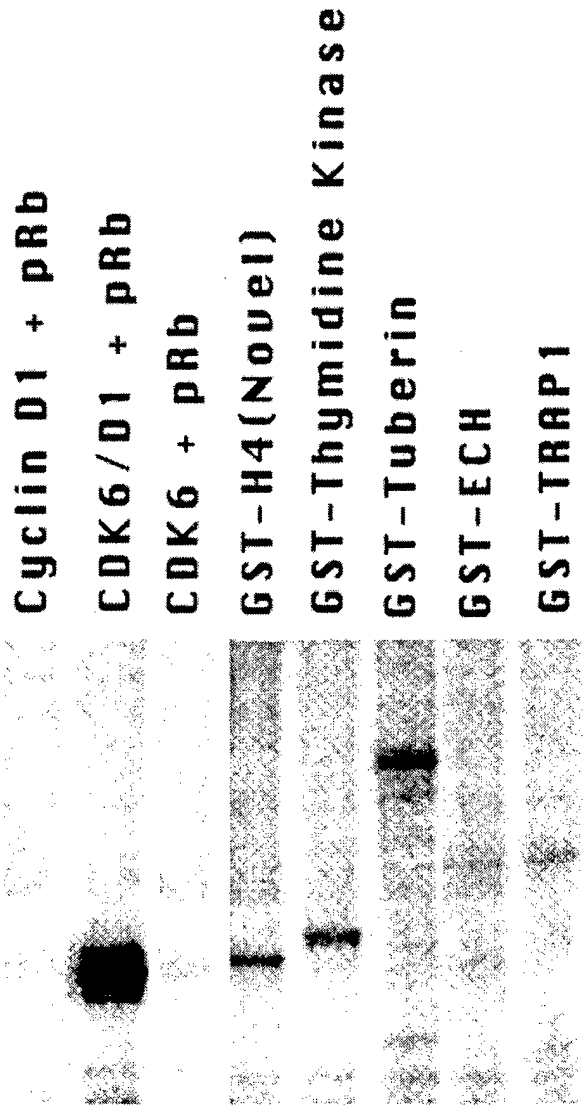

Figure 6. **In Vitro Kinase assay.**
Several isolates from the pGBT7-mtCDK6/D1 three-hybrid screen were expressed as GST-fusion proteins and isolated from BL21(DE3), a protease deficient strain of E. coli., using GST-beads. The isolated fusion proetins were then incubated with baculovirus extracts expressing either CDK6 and Cyclin D1, in the presence of gamma-32P. The samples were run on a 12.5% SDS polyacrylamide gel. The gel was analyzed on a Storm380 Phosphorimager after exposure for 8 hours.